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Molecular Analysis of and Identification of Antibiotic Resistance Genes in Clinical Isolates of *Salmonella typhi* from India

PHILIPPA M. A. SHANAHAN,[1] MARY V. JESUDASON,[2] CHRISTOPHER J. THOMSON,[1] AND SEBASTIAN G. B. AMYES[1]*

Department of Medical Microbiology, University of Edinburgh, Edinburgh EH8 9AG, United Kingdom,[1] and Department of Clinical Microbiology, Christian Medical College and Hospital, Vellore-632 004, India[2]

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A representative sample of 21 *Salmonella typhi* strains isolated from cultures of blood from patients at the Christian Medical College and Hospital, Vellore, India, were tested for their susceptibilities to various antimicrobial agents. Eleven of the *S. typhi* strains possessed resistance to chloramphenicol (256 mg/liter), trimethoprim (64 mg/liter), and amoxicillin (>128 mg/liter), while four of the isolates were resistant to each of these agents except for amoxicillin. Six of the isolates were completely sensitive to all of the antimicrobial agents tested. All the *S. typhi* isolates were susceptible to cephalosporin agents, gentamicin, amoxicillin plus clavulanic acid, and imipenem. The antibiotic resistance determinants in each *S. typhi* isolate were encoded by one of four plasmid types. Plasmid-mediated antibiotic resistance genes were identified with specific probes in hybridization experiments; the genes responsible for chloramphenicol, trimethoprim, and ampicillin resistance were chloramphenicol acetyltransferase type I, dihydrofolate reductase type VII, and TEM-1 β-lactamase, respectively. Pulsed-field gel electrophoresis analysis of *Xba*I-generated genomic restriction fragments identified a single distinct profile (18 DNA fragments) for all of the resistant isolates. In comparison, six profiles, different from each other and from the resistance profile, were recognized among the sensitive isolates. It appears that a single strain containing a plasmid conferring multidrug-resistance has emerged within the *S. typhi* bacterial population in Vellore and has been able to adapt to and survive the challenge of antibiotics as they are introduced into clinical medicine.

Typhoid fever is distressingly prevalent in developing countries, where it remains a major health problem (3, 39). The annual global incidence of this disease has been estimated to be 21 million cases, with more than 700,000 deaths (36). Infection with *Salmonella typhi*, the causative organism of this disease, requires effective antimicrobial chemotherapy in order to reduce mortality (8). Chloramphenicol was the "gold standard" agent for the treatment of this infection (17), but with the emergence of chloramphenicol-resistant strains, ampicillin and trimethoprim were considered suitable alternatives (8). Since 1989, however, multidrug-resistant (MDR) *S. typhi* strains that are no longer susceptible to these three first-line antibiotics have emerged (18, 37). Indeed, these MDR *S. typhi* strains have become a serious problem globally and have been reported not only in the Indian subcontinent but also in Latin America, Egypt, Nigeria, China, Korea, Vietnam, and the Philippines (27). As a result, the potential of other antimicrobial agents including broad-spectrum cephalosporins and fluoroquinolones for the treatment of typhoid fever have been investigated (13, 21).

Antibiotic resistance in *S. typhi* is often plasmid mediated. In particular, resistance to chloramphenicol, ampicillin, trimethoprim, sulfonamides, and tetracycline is often encoded by plasmids belonging to the incompatibility complex group *Inc*HI (31). These plasmids are large (~180 kb) and conjugative and originate from Southeast Asia (16, 37).

Until recently, it had been suggested that, with few exceptions, *S. typhi* represented a single clone with little intraspecies divergence (29). New molecular biology-based techniques, however, in particular pulsed-field gel electrophoresis (PFGE), are extremely discriminatory and indicate genetic heterogeneity among *S. typhi* isolates (24, 35, 36). Use of this technique for the fingerprinting of each strain provides a tool that can successfully be used in the epidemiological investigation of *S. typhi* outbreaks.

Between 1990 and 1994 MDR *S. typhi* strains with reduced susceptibilities to the fluoroquinolones (4) were isolated in Vellore, in southern India, as the cause of epidemic typhoid. Concurrently, chloramphenicol-sensitive *S. typhi* strains continued to be isolated, suggesting that both varieties are endemic (19). This unusual phenomenon prompted this investigation. In this paper, the antibiotic resistance levels in *S. typhi* are reported, the genes associated with the antibiotic resistance are identified, and the isolates are typed at the molecular level and compared with a coexisting subpopulation of chloramphenicol-sensitive *S. typhi.*

MATERIALS AND METHODS

Bacterial strains. *S. typhi* strains were isolated at the Christian Medical College and Hospital, Vellore, India, between 1992 and 1994. MDR *S. typhi* strains were defined as those strains possessing chloramphenicol, ampicillin, and trimethoprim resistance. Each isolate was confirmed as being *S. typhi* with API 20E test strips (BióMerieux, Marcy l'Etoile, France).

Sensitivity testing. The MICs of chloramphenicol, trimethoprim, amoxicillin, amoxicillin plus clavulanic acid, cefotaxime, and imipenem were determined as described previously (25). For antibiogram analysis the same method as that used for the MIC determinations was used, except that a fixed concentration of antimicrobial agent was incorporated into the Iso-Sensitest agar (Oxoid, Basingstoke, United Kingdom) plates.

PFGE. Genomic DNA was prepared as described by Butler et al. (5). DNA restricted with *Xba*I (TCTAGA) was separated by PFGE by using a CHEF DR II system (Bio-Rad) at 14°C for 22 h at 200 V with a pulse time of 1 to 60 s. The

* Corresponding author. Mailing address: Department of Medical Microbiology, University of Edinburgh, Teviot Place, Edinburgh EH8 9AG, United Kingdom. Phone: 44 131 650 3163. Fax: 44 131 650 6882. E-mail: s.g.b.amyes@ed.ac.uk.

TABLE 1. MICs of a range of antibiotics for MDR and chloramphenicol-sensitive *S. typhi* strains

Strain type and no.	MIC (mg/liter)					
	Chloramphenicol	Trimethoprim	Amoxicillin	Amoxicillin-clavulanic acid	Cefotaxime	Imipenem
Range of MICs	0.25–128	0.032–32	0.25–128	0.25–128	0.004–128	0.125–32
MDR isolates						
ST1	>128	>32	>128	4	0.0625	0.125
ST2	>128	>32	>128	4	0.0625	0.125
ST3	>128	>32	0.5	0.5	0.0625	0.125
ST4	>128	>32	>128	4	0.0625	0.125
ST5	>128	>32	0.5	0.5	0.0625	0.125
ST6	>128	>32	>128	4	0.0625	0.125
ST7	>128	>32	0.5	0.5	0.0625	0.125
ST8	>128	>32	>128	4	0.0625	0.125
ST9	>128	>32	>128	4	0.0625	0.125
ST10	>128	>32	>128	4	0.0625	0.125
ST11	>128	>32	>128	4	0.0625	0.125
ST12	>128	>32	0.5	0.5	0.0625	0.125
ST13	>128	>32	>128	4	0.0625	0.125
ST14	>128	>32	>128	4	0.0625	0.125
ST15	>128	>32	>128	4	0.0625	0.125
Chloramphenicol-sensitive isolates						
ST45	2	0.0625	0.5	0.5	0.0625	0.125
ST46	2	0.125	0.5	0.5	0.0312	0.125
ST48	2	0.0625	0.5	0.5	0.0312	0.125
ST49	2	0.0312	0.5	0.5	0.0312	0.125
ST51	2	0.0625	1	1	0.0625	0.125
ST52	2	0.0312	0.5	0.5	0.0312	0.125

similarity between two restriction fragment length polymorphisms was scored with the coefficient of similarity (*F*) or the Dice coefficient (9), in which an *F* value of 1.0 indicates that two isolates have identical PFGE patterns.

Conjugational transfer of drug resistance and plasmid analysis. Conjugation experiments with the MDR *S. typhi* strains were performed by the method of Amyes and Gould (2). The conjugations were performed for 18 h at 28 and 37°C. Plasmids were isolated by a modification of the method described by Takahashi and Nagano (34). The plasmid DNA was digested for 2 h at 37°C with 10 U of *Eco*RI restriction endonuclease according to the manufacturer's instructions (Gibco BRL, Paisley, United Kingdom). A repeat digest of this plasmid DNA was performed under the same conditions described above but with 10 U of *Mlu*I restriction endonuclease (Gibco BRL). *Mlu*I was chosen specifically because this restriction enzyme does not cut within the TEM-1, dihydrofolate reductase (DHFR) type VII, or the chloramphenicol acetyltransferase type I (CAT-I) gene. The digested DNA was analyzed in each case by electrophoresis on a 0.6% agarose gel at 50 V for 21 h.

DNA hybridizations. Dot blots and Southern blots of the plasmid DNA from the transconjugants were prepared on a transfer membrane (Hybond N^+) as instructed by the manufacturer (Amersham International plc, Little Chalfont, United Kingdom). Control plasmids encoding the type Ia, Ib, V, and VII DHFRs (1) and CAT-I, -II, and -III (all provided by Kevin Towner, University of Nottingham, Nottingham, United Kingdom), and plasmids R1 (14) and R1010 (28) encoding the TEM-1 and SHV-1 β-lactamases, respectively, were used.

Oligonucleotide probes for distinguishing between different DHFR genes were used as described by Adrian et al. (1). A heterogeneous sequence that occurs throughout the same region of the CAT-I, -II, and -III genes was selected for the construction of a 30-base oligonucleotide probe specific for the CAT-I gene, as follows: 5′-TATGTGTAGAAACTGCCGGAAATCGTCGTG. This probe was tested for homology with other DNA sequences in the GenBank database. A TEM-1 gene probe was prepared from a TEM-derived PCR product generated with oligonucleotide primers described previously (6). Hybridizations were carried out with either an ECL 3′ oligo labelling kit or a random prime labelling and detection kit according to the manufacturer's recommendations (Amersham International plc). Stringency washes were performed as described previously (15).

β-Lactamase analysis. β-Lactamases from the transconjugants were isolated and investigated by isoelectric focusing and biochemical analysis as described elsewhere (25).

PCR amplification for incompatibility testing. A 365-bp region of the RepHI1A replicon was amplified from plasmid DNA by use of a *Taq* polymerase kit obtained from Gibco BRL. The final volume in the tubes for amplification was 100 μl and consisted of 10× *Taq* PCR buffer, 2.5 mM $MgCl_2$, each deoxynucleoside triphosphate at a concentration of 200 μM, 10 pmol of each primer (5′-GGTCCAACCCATTGCTTTAC and 5′-CACGGAAAGAAATCACAAC, as recommended by Gabant et al. [11] and purchased from Oswel DNA Service, University of Southampton), 0.1 μg of DNA, and 2 U of *Taq* polymerase. The amplification reaction, conducted in a Techne thermocycler, consisted of 30 cycles at 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s. A final extension step ran at 72°C for 10 min.

RESULTS

Bacterial strains. A total of 21 *S. typhi* isolates from Vellore, India, were investigated in the study: 15 MDR *S. typhi* strains and 6 chloramphenicol-sensitive *S. typhi* strains.

Antimicrobial sensitivity testing. As determined previously (4), none of the isolates were clinically resistant to ciprofloxacin. All of the chloramphenicol-sensitive *S. typhi* strains were sensitive to all the antimicrobial agents tested, namely, chloramphenicol, trimethoprim, amoxicillin, amoxicillin plus clavulanic acid, cefotaxime, and imipenem (Table 1). In contrast, all of the MDR isolates were resistant to chloramphenicol (MIC, 256 mg/liter) and trimethoprim (MIC, 64 mg/liter). Resistance to ampicillin (MIC, >128 mg/liter) was detected in 11 of these isolates; isolates ST3, ST5, ST7, and ST12 were sensitive to this agent. All the isolates were susceptible to amoxicillin plus clavulanic acid, cefotaxime, and imipenem.

Molecular typing. After digestion of the chromosomal DNA from each of the MDR *S. typhi* strains with *Xba*I, a single restriction endonuclease analysis (REA) pattern, which consisted of 18 distinct DNA fragments, was generated by PFGE (Fig. 1). In contrast, after digestion of the chromosomal DNA of the chloramphenicol-sensitive *S. typhi* strains with *Xba*I, six separate REA patterns were produced by PFGE (Fig. 1). The *F* values for these isolates were found to range between 0.68 and 0.93. When compared with the MDR *S. typhi* profile, the *F* values of each of the chloramphenicol-sensitive *S. typhi* isolates ranged from 0.66 to 0.88. Each REA pattern produced by PFGE was confirmed to be stable and reproducible with repeated digestion of the genomic DNA preparations.

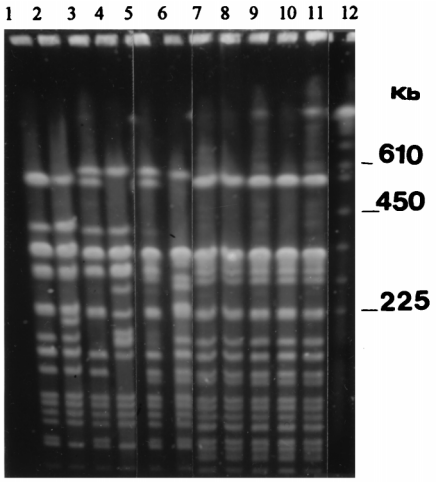

FIG. 1. PFGE of *Xba*I-digested chloramphenicol-sensitive and representative MDR *S. typhi* strains. Lanes: 1, ST45; 2, ST46; 3, ST48; 4, ST49; 5, ST51; 6, ST52; 7, ST1; 8, ST2; 9, ST3; 10, ST4; 11, ST5; 12, *Saccharomyces cerevisiae* standard.

Conjugational transfer of drug resistance and plasmid analysis. Sensitivity testing identified all of the MDR isolates as trimethoprim resistant. This agent was therefore used for selection in the conjugation studies. It is established that the transfer of antibiotic resistance genes in *Salmonella* spp. occurs more readily at lower temperatures. It was not surprising, therefore, that each strain exhibited the ability to transfer trimethoprim resistance into *Escherichia coli* J62-2 at 28°C (Table 2).

For each of the 15 transconjugants, the MICs of chloramphenicol and trimethoprim were 128 mg/liter. For 10 of the transconjugants amoxicillin MICs were 128 mg/liter. The plasmids which originated in ST3, ST5, ST7, and ST12 possessed no resistance to amoxicillin. Interestingly, ST14, which was amoxicillin resistant, contained a plasmid encoding no resistance to this agent. As determined by the breakpoint value, all the transconjugants were resistant to tetracycline and spectinomycin, and in addition, the amoxicillin-resistant transconjugants also possessed resistance to sulfamethoxazole and streptomycin (Table 2).

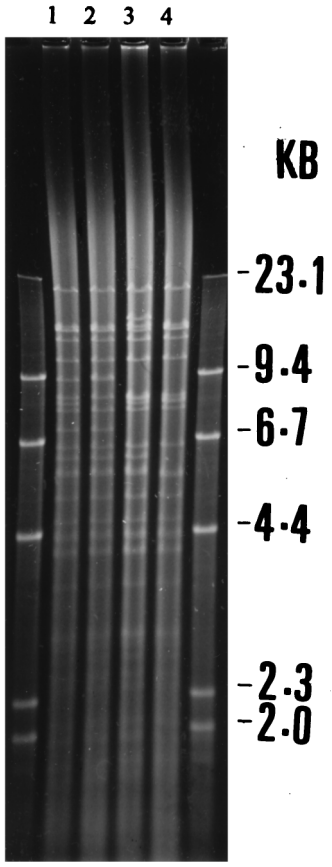

FIG. 2. DNA from representative plasmids restricted with *Eco*RI on a 0.6% agarose gel. Lanes: 1, group A; 2, group B; 3, group D; 4, group C.

TABLE 2. *S. typhi* isolations, antibiogram profiles, and transfer of resistance determinants into *E. coli* J62-2

Strain no.	Yr of isolation	Temp (°C) of transfer into *E. coli* J62-2	Plasmid group	Transferable resistance determinants[a]
ST1	1994	28	B	Cm Tp Ap Sm Sx Tc Sp
ST2	1994	28	B	Cm Tp Ap Sm Sx Tc Sp
ST3	1992	28 and 37	C	Cm Tp Tc Sp
ST4	1994	28 and 37	B	Cm Tp Ap Sm Sx Tc Sp
ST5	1992	28	C	Cm Tp Tc Sp
ST6	1994	28	B	Cm Tp Ap Sm Sx Tc Sp
ST7	1993	28	D	Cm Tp Tc Sp
ST8	1994	28 and 37	B	Cm Tp Ap Sm Sx Tc Sp
ST9	1994	28 and 37	A	Cm Tp Ap Sm Sx Tc Sp
ST10	1994	28 and 37	A	Cm Tp Ap Sm Sx Tc Sp
ST11	1994	28 and 37	B	Cm Tp Ap Sm Sx Tc Sp
ST12	1992	28 and 37	C	Cm Tp Tc Sp
ST13	1994	28	B	Cm Tp Ap Sm Sx Tc Sp
ST14	1994	28	D	Cm Tp Tc Sp
ST15	1994	28 and 37	B	Cm Tp Ap Sm Sx Tc Sp
ST45	1993			
ST46	1993			
ST48	1994			
ST49	1994			
ST51	1994			

[a] The MICs of chloramphenicol (Cm; MIC, 128 mg/liter); trimethoprim (Tp; MIC, 128 mg/liter), and ampicillin (Ap; MIC, 128 mg/liter) were determined. Breakpoint values for streptomycin (Sm; 10 mg/liter), sulfamethoxazole (Sx; 32 mg/liter), tetracycline (Tc; 8 mg/liter), and spectinomycin (Sp; 10 mg/liter) were determined.

Among the 15 plasmids, four different plasmid profiles were identified after digestion with the *Eco*RI restriction endonuclease (Fig. 2). Plasmids with identical restriction fragment length polymorphisms were allocated to the same group. Digestion with the *Mlu*I restriction endonuclease (Fig. 3) confirmed the presence of four plasmid groups designated groups A to D. Two plasmids, 160 kb in size, were present in group A. These plasmids originated in strains isolated in 1994. Group B contained eight plasmids which were calculated as being 150 kb in size, and all originated in strains isolated in 1994. The three plasmids in group C, all originating in strains isolated in 1992, were 170 kb and mediated no resistance to ampicillin. Finally, two plasmids were allocated to group D and were calculated as being 140 kb in size. These plasmids originated in strains isolated in 1993 and 1994 and, like group C, did not mediate resistance to ampicillin (Table 2).

Gene detection. Isoelectric focusing of each of the β-lactamase preparations from the ampicillin-resistant transconjugants identified the presence of an enzyme that cofocused with the TEM-1 β-lactamase control at a pI value of 5.4. No extended-spectrum activity was possessed, as established by hy-

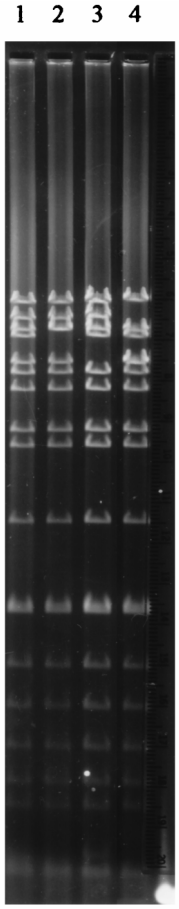

FIG. 3. DNA from representative plasmids restricted with *Mlu*I on a 0.6% agarose gel. Lanes: 1, group A; 2, group B; 3, group D; 4, group C.

drolysis assays. Positive plasmid DNA dot blot hybridizations with a TEM-1 gene probe confirmed its presence (Fig. 4A).

DNA dot blot hybridizations with the DHFR and CAT oligonucleotide probes indicated that each plasmid, regardless of the restriction endonuclease profile, encoded the *dfrVII* and the *cat-1* genes, respectively. Southern blots of each plasmid digested with the *Mlu*I restriction endonuclease indicated that for each of the four different plasmid types, the two oligonucleotide probes and the gene probe all positively hybridized to DNA fragments of the same size (Fig. 4B and C). None of the group C or group D plasmids possessed ampicillin resistance, and correspondingly, there was no hybridization with the TEM-1 gene probe (Fig. 4A).

Incompatibility group testing. The incompatibility group of the plasmids isolated from each of the transconjugants was determined by PCR. In each case, a 365-bp region of the RepHI1A replicon was amplified, providing evidence that each transconjugant contained a plasmid belonging to incompatibility group *Inc*HI1.

DISCUSSION

There has been increasing concern about the prevalence of MDR *S. typhi* strains that are insusceptible to chloramphenicol, ampicillin, and trimethoprim (23, 38). Indeed there is an urgent need to examine the status of resistant *S. typhi* so that a rational approach to therapy may be adopted. In this study, we investigated 21 *S. typhi* isolates, obtained from patients with typhoid fever in Vellore, India, between 1992 and 1994. MIC determinations indicated that 15 of the isolates were resistant to chloramphenicol and trimethoprim. While high-level resistance to ampicillin predominated among 11 *S. typhi* strains, strains ST3, ST5, ST7, and ST12 were completely sensitive to this agent. It has been suggested that the emergence of chloramphenicol-resistant strains of *S. typhi* may be a result of the indiscriminate use of this agent and the use of this agent in irrational combinations (32). This is also the probable explanation for the emergence of trimethoprim and ampicillin resistance.

In response to the emergence of multiantibiotic-resistant *S. typhi*, a number of studies have investigated the efficacies of newer compounds including expanded-spectrum cephalosporins and fluoroquinolones (13, 21). Specifically, ceftriaxone has been very successful, with low rates of fever relapse, but this agent, like other expanded-spectrum cephalosporins, including cefotaxime and ceftazidime, is hindered by its expense and the need for parenteral administration (26). The MIC results in the current investigation revealed that *S. typhi* is sensitive to expanded-spectrum cephalosporins, suggesting that, at present, these drugs may remain clinically effective. It should be remembered, however, that the appearance in other gram-negative species of extended-spectrum β-lactamases possessing resistance to the later cephalosporins was a direct result of the extensive use of these agents in the hospital environment (7).

Studies investigating clinical isolates of *S. typhi* in Vellore suggest the coexistence of two populations of organisms: those which are chloramphenicol sensitive and those which are MDR (19). The epidemiology of these MDR *S. typhi* strains was elucidated after PFGE, which allows differentiation of the strains. In this study, *Xba*I, an enzyme that recognizes the rare tetranucleotide CTAG which is counterselected in many bacterial genomes, was used (30). In previous studies that have investigated *S. typhi* strain variation, this enzyme has been used and has produced clear REA patterns with approximately 20 fragments. Furthermore, these studies have established that sporadic outbreaks of typhoid fever are associated with heterogeneous isolates of *S. typhi* (24, 36). In the current study, a single REA pattern of 18 fragments was identified in all the MDR isolates, indicating the clonal spread of this resistant strain of *S. typhi* through the community in Vellore. Interestingly, genetic variation clearly existed between MDR *S. typhi* isolates and the *S. typhi* isolates from the chloramphenicol-sensitive subpopulation. It is unclear if this MDR strain type has a particular predisposition for the acquisition of plasmids encoding antibiotic resistance genes.

Previous studies have revealed plasmid-mediated antibiotic resistance in *S. typhi* (10, 12, 20, 22, 37). Similarly, in the current investigation each of these resistance determinants was transferable to a standard laboratory host strain. More recent reports suggest that these plasmids, which belong to the *Inc*HI incompatibility group, frequently encode resistance to chloramphenicol, trimethoprim, ampicillin, sulfonamides, and tetracyclines, and have been estimated as being between 110 and 120 mDa (165 and 180 kb) (37). The plasmids detailed in the current investigation were also found to belong to the *Inc*HI group, specifically *Inc*HI1, and were calculated as being between 140 and 170 kb.

This is the first investigation which has identified the particular genes responsible for plasmid-mediated antibiotic resistance in *S. typhi*. The identification of a TEM-1 group β-lactamase as the determinant of β-lactam resistance in the *S. typhi* isolates is perhaps not surprising because this β-lactamase has been found extensively among other clinical isolates. The clin-

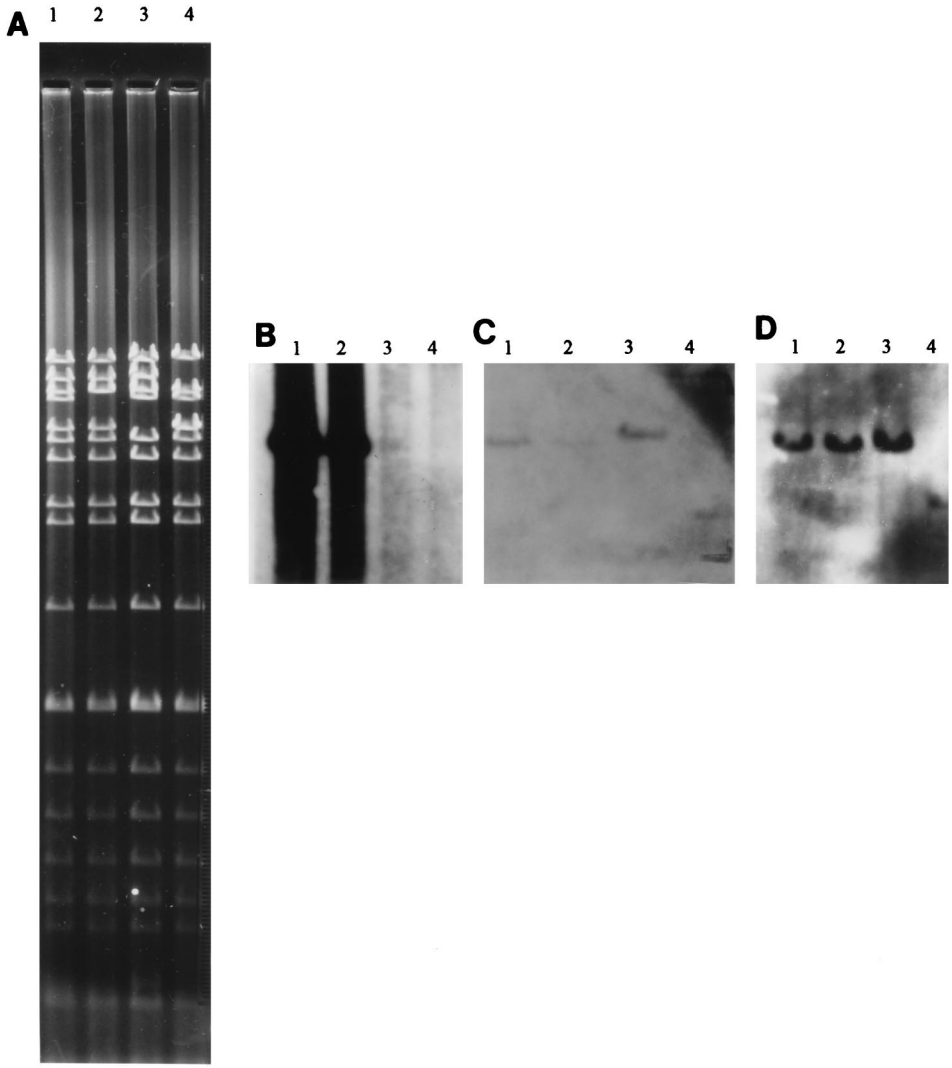

FIG. 4. DNA from representative plasmids restricted with the *Mlu*I restriction endonuclease on a 0.6% agarose gel (A). Southern blots, prepared from panel A, show the DNA fragments from representative plasmids which hybridized to the TEM-1 β-lactamase gene probe (B), the type VII DHFR oligonucleotide probe (C), and the CAT-I oligonucleotide probe (D). Lanes: 1, plasmid group A; 2, plasmid group B; 3, plasmid group D; 4, plasmid group C.

ical implications of the presence of TEM-1 is of concern because this β-lactamase is recognized as the progenitor to many extended-spectrum β-lactamases and inhibitor-resistant β-lactamases.

Because of the high degree of homology found in the conserved regions of the DHFRs, it is vital that specific oligonucleotide probes be used in order to distinguish between the different DHFRs (1). The identification of the plasmid-encoded type VII DHFR in *S. typhi* confirms the ubiquitous distribution of this particular DHFR. This enzyme has already been isolated in Sweden, Finland, Nigeria, Sri Lanka, the United Kingdom, and more recently, South Africa (33).

As far as we are aware, no oligonucleotide probes have been used in the screening of plasmid-mediated chloramphenicol resistance among members of the family *Enterobacteriaceae*. It is difficult, therefore, to establish the incidence of this particular group of enzymes. Among the isolates screened, only the *cat1* gene was identified.

We may conclude that in Vellore a specific strain of *S. typhi* has been established and has persisted in the bacterial population. Furthermore, through the acquisition of a plasmid conferring MDR, this individual strain has undergone the necessary and appropriate adaptation for survival in the changing antibiotic environment.

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